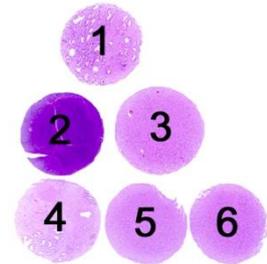


**α-methylacyl-CoA racemase (AMACR, P504S) / Prostate intraepithelial neoplasia (PIN) cocktail**

The slide to be stained for AMACR/PIN-cocktail comprised:

1. Prostate intraepithelial neoplasia (PIN), 2. Tonsil, 3. Kidney, 4. Prostate hyperplasia, 5- 6. Prostate carcinoma.

All tissues were fixed in 10% neutral buffered formalin.



Criteria for assessing an AMACR staining as optimal included:

- A moderate to strong granular cytoplasmic staining of the epithelial cells lining the renal proximal tubules.
- A moderate to strong cytoplasmic staining of the majority of the neoplastic cells of the PIN and the two prostate adenocarcinomas.
- A negative or only weak focal cytoplasmic staining of the epithelial cells of the hyperplastic prostate glands.
- A negative or only weak cytoplasmic staining of the stromal cells.

In case of using a PIN cocktail the criteria also included:

AMACR + p63:

- A moderate to strong, distinct nuclear staining in almost all squamous epithelial cells in the tonsil and in the basal cells of the prostate hyperplastic glands and the PIN lesion.

AMACR + p63 + CK high molecular weight (HMW: CK5, CK5/6, CK14)

- A moderate to strong, distinct nuclear and cytoplasmic staining in almost all squamous epithelial cells in the tonsil and in the basal cells of the prostate hyperplastic glands and PIN lesion.

106 laboratories participated in this assessment. 70 used AMACR as a single marker out of which 90 % achieved a sufficient mark. 36 used a PIN-cocktail out of which 89 % achieved a sufficient mark.

In table 1 the antibodies (Abs) for AMACR and marks are summarized.

Table 1. **Abs and scores for AMACR, run 26**

Concentrated Abs	N	Vendor	Optimal	Good	Borderl.	Poor	Suff. <sup>1</sup>	Suff. OPS <sup>2</sup>
rmAb clone <b>13H4</b>	45	Dako	34	17	4	1	91 %	96 %
	5	Immunologic						
	3	Biologo						
	2	NeoMarkers						
	1	Master Diagnostica						
pAb <b>p504S (CP200)</b>	6	BioCare	2	3	1	0	83 %	100 %
<b>Ready-To-Use Abs</b>								
rmAb clone <b>13H4, IRO60</b>	5	Dako	5	0	0	0	100 %	100 %
rmAb clone <b>13H4, RM-9130-R7</b>	2	NeoMarkers	1	0	1	0	-	-
rmAb clone <b>13H4, 504R-17</b>	1	Cell Marque	0	1	0	0	-	-
pAb <b>p504S, PP200</b>	1	Biocare	0	1	0	0	-	-
<b>Total</b>	71		43	21	6	1	-	-

<b>Proportion</b>		61 %	29 %	9 %	1 %	90 %	95 %
-------------------	--	------	------	-----	-----	------	------

1) Proportion of sufficient stains (optimal or good)

2) Proportion of sufficient stains with optimal protocol settings only, see below.

### Concentrated Abs

rmAb **13H4**: The protocols giving an optimal result were all based on heat induced epitope retrieval (HIER) using Tris-EDTA/EGTA pH 9 (13/18)\*, Target Retrieval Solution pH 9 (EnVision FLEX TRS high pH, Dako)(9/11), Cell Conditioning 1 (BenchMark, Ventana) (4/14), EDTA/EGTA pH 8 (1/2), Bond Epitope Retrieval Solution 2 (Bond, Leica) (4/4) or Citrate pH 6 (3/7) as retrieval buffer. The rmAb was typically diluted in the range of 1:25 – 1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings 51 out of 53 (96 %) laboratories produced a sufficient staining (optimal or good).

\* (number of optimal results/number of laboratories using this buffer)

pAb **p504S, CP200**: The protocols giving an optimal result were all based on HIER using Tris-EDTA/EGTA pH 9 (1/2) or Target Retrieval Solution pH 9 (EnVision FLEX TRS high pH, Dako) (1/1). The pAb was typically diluted in the range of 1:25 – 1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings both of 2 laboratories produced a sufficient staining.

### Ready-To-Use Abs

rmAb clone **13H4**, prod. no IR060, Dako: The protocols giving an optimal result were all based on HIER using Target Retrieval Solution pH 9 (EnVision FLEX TRS high pH), an incubation time of 20 or 30 min in the primary Ab and EnVision Flex/Flex+(K8000/K8002) as the detection system. Using these protocol settings 5 out of 5 (100 %) laboratories produced a sufficient staining.

rmAb clone **13H4**, prod. no. RM-9130-R7, NeoMarkers: The protocol giving an optimal result was based on HIER using mild Cell Conditioning 1 (BenchMark, Ventana), an incubation time of 32 min in the primary Ab and iView (760-091) as the detection system. Using these protocol settings 1 out of 2 laboratories produced a sufficient staining.

rmAb clone **13H4**, prod. no. 504R-17, Cell Margue: The protocol giving an optimal result was based on HIER using standard Cell Conditioning 1 (BenchMark, Ventana), an incubation time of 32 in the primary Ab and Ultra View (760-500) as the detection system.

In table 2 the antibodies (Abs) for a PIN-cocktail and marks are summarized.

Table 2. **Abs and scores for PIN-cocktail, run 26**

<b>Concentrated Abs 2 Abs AMACR+p63</b>	<b>N</b>	<b>Vendor</b>	<b>Optimal</b>	<b>Good</b>	<b>Borderl.</b>	<b>Poor</b>	<b>Suff.<sup>1</sup></b>	<b>Suff. OPS<sup>2</sup></b>
rmAb clone <b>13H4</b> + mAb clone <b>4A4</b>	2	Biologo	0	1	1	0	-	-
rmAb clone <b>13H4</b> + mAb clone <b>4A4</b>	10	Dako/Dako Cell Marque/Cell Marque Zeta Corporation/Ventana	6	5	1	0	90 %	100 %
rmAb clone <b>13H4</b> + mAb clone ( <b>4A4&amp;Y4A3</b> )	2	Dako/NeoMarkers	0	1	1	0	-	-
<b>Concentrated Abs 3 Abs AMACH+p63+CK HMW</b>								
rmAb clone <b>13H4</b> + mAb clone <b>4A4</b> + mAb clone <b>D5/16 B4</b>	5	Dako/Dako/Dako	3	2	0	0	100 %	100 %
rmAb clone <b>13H4</b> + mAb clone ( <b>4A4&amp;Y4A3</b> ) + mAb clone <b>D5/16 B4</b>	1	Dako/NeoMarkers/Dako	1	0	0	0	-	-
rmAb clone <b>13H4</b> + mAb clone <b>4A4</b> + mAb clone <b>XM26</b>	1	Biologo/DBS/DBS	1	0	0	0	-	-
rmAb clone <b>13H4</b> + mAb clone <b>4A4</b> + mAb clone <b>34BE12</b>	1 1	Dako/Dako/Dako Dako/NeoMarkers/Dako	1	0	1	0	-	-
<b>Ready-To-Use Abs 2Abs AMACR+p63</b>								

mAb clone <b>4A4</b> + pAb <b>P504S, PPM201,</b> <b>VP201</b>	6	BioCare	3	3	0	0	100 %	100 %
rmAb clone <b>13H4</b> + mAb clone <b>4A4, PIN001-G</b>	1	Biologo	0	1	0	0	-	-
rmAb clone <b>13H4</b> + mAb clone <b>4A4,</b> <b>Z2006</b>	1	Zeta Corporation	0	1	0	0	-	-
<b>Ready-To-Use Abs 3Abs</b> <b>AMACH+p63+CK HMW</b>								
rmAb clone <b>13H4</b> + mAb clone <b>4A4</b> + mAb clone <b>34BE12,</b> <b>PIN002-G</b>	1	Biologo	1	0	0	0	-	-
<b>Ready-To-Use Abs 4Abs</b> <b>AMACH+p63+CK HMW</b>								
pAb <b>P504S</b> + mAb clone <b>4A4</b> + mAb clone <b>XM26</b> + mAb clone <b>LL002,</b> <b>PPM225DSAA (PIN4)</b>	1	BioCare	1	0	0	0	-	-
<b>Total</b>	35		17	14	4	0	-	-
<b>Proportion</b>			49 %	40 %	11 %	0 %	89 %	-

1) Proportion of sufficient stains (optimal or good)

2) Proportion of sufficient stains with optimal protocol settings only, see below.

Following central protocol parameters were used to obtain an optimal staining:

#### **In-house PIN-cocktails based on concentrated Abs**

rmAb clone **13H4** + mAb clone **4A4**: The protocols giving an optimal result were all based on heat induced epitope retrieval (HIER) using Tris-EDTA/EGTA pH 9 (2/3)\*, Target Retrieval Solution pH 9 (EnVision FLEX TRS high pH, Dako) (2/2), or Cell Conditioning 1 (BenchMark, Ventana) (2/4) as retrieval buffer. The rmAb clone 13H4 was typically diluted in the range of 1:25 – 1:1,000, whereas the mAb clone 4A5 was diluted 1:50 – 1:1,000 depending on the total sensitivity of the protocol employed. Using these protocol settings 10 out of 10 (100 %) laboratories produced a sufficient staining (optimal or good).

\* (number of optimal results/number of laboratories using this buffer)

rmAb clone **13H4** + mAb clone **4A4** + mAb clone **D5/16 B4**: The protocols giving an optimal result were all based on HIER using Tris-EDTA/EGTA pH 9 (1/2)\*, Target Retrieval Solution pH 9 (EnVision FLEX TRS high pH, Dako) (1/2), or Cell Conditioning 1 (BenchMark, Ventana) (1/1) as retrieval buffer. The rmAb clone 13H4 was typically diluted in the range of 1:400 – 800, mAb clone 4A4 1:200 – 1:800 and the clone D5/16 B4 1:100 – 1:800 depending on the total sensitivity of the protocol employed. Using these protocol settings 5 out of 5 (100 %) laboratories produced a sufficient staining (optimal or good).

rmAb clone **13H4** + mAb clone (**4A4 + Y4A3**) + mAb clone **D5/16 B4**: The protocol giving an optimal result was based on HIER using Tris-EDTA/EGTA pH 9 as retrieval buffer. The rmAb/mAb/mAb was diluted 1:200/1:4.000/1:100.

rmAb clone **13H4** + mAb clone **4A4** + mAb clone **XM26**: The protocol giving an optimal result was based on HIER using Cell Conditioning 1 (BenchMark, Ventana) as retrieval buffer. The rmAb/mAb/mAb was diluted 1:100/1:100/1:100.

rmAb clone **13H4** + mAb clone **4A4** + mAb clone **34BE12**: The protocol giving an optimal result was based on HIER using Cell Conditioning 1 (BenchMark, Ventana) as retrieval buffer. The rmAb/mAb/mAb was diluted 1:50/1:25/1:100.

#### **PIN-cocktails based on Ready-To-Use Abs**

mAb clone 4A4 +pAb P504S, prod. no. PPM201, BioCare: The protocols giving an optimal result were all based on HIER using Tris-EDTA/EGTA pH 9 (2/2)\* or Target Retrieval Solution pH 9 (EnVision FLEX TRS high pH, Dako) (1/2) an incubation time of 20 or 25 min in the primary Ab and EnVision (K8002/K5007) as the detection system. Using these protocol settings 4 out of 4 laboratories produced a sufficient staining.

\* (number of optimal results/number of laboratories using this buffer)

rmAb clone **13H4** + mAb clone **4A4** + mAb clone **34BE12**, prod. no. PIN002-G, Biologo: The protocol giving an optimal result was based on HIER using Target Retrieval Solution pH 9 (EnVision FLEX TRS high pH, Dako) an incubation time of 60 min in the primary Ab and EnVision (K5007) as the detection system.

mAb clone **4A4** + mAb clone **XM26** + mAb clone **LL002** + pAb **P504S**, prod. no. PPM225DSAA (PIN4), BioCare: The protocol giving an optimal result was based on HIER using Tris-EDTA/EGTA pH 9 an incubation time of 30 min in the primary Ab and MACH2 double stain as the detection system.

The most frequent causes of insufficient stains were:

- Too low or too high concentration of the primary Ab for AMACR
- Too low concentration of the primary Ab for p63 in an in house PIN cocktail

In this assessment and in concordance to the previous assessment of AMACR the prevalent feature of an insufficient staining was a too weak or false negative staining of the epithelial cells in the prostate carcinomas and PIN lesion primarily due to a too low concentration of the primary Ab. In only 2 stains, the insufficient result was caused by a false positive reaction (diffuse background reaction and cytoplasmic staining of the normal prostate epithelial cells). In order to identify a correct calibration of the immunohistochemical protocol for AMACR, kidney appeared to be a suitable control: In the optimally calibrated protocols virtually all the epithelial cells of the proximal tubules showed a strong and distinct granular staining, indicating that these cells may serve as a reliable positive critical stain quality indicator (CSQI) for AMACR. However, kidney is only a reliable positive control for AMACR, when a non-biotin based detection system is applied (as the non-specific reaction of endogenous biotin in the renal tubules mimics the specific staining reaction). As negative control for AMACR, a normal prostate is recommendable: The epithelial cells must be negative or only show a focal staining and the stromal cells must be negative.

In the assessment 35 laboratories used a PIN-cocktail based on 2 – 4 markers. All were based on AMACR and p63, and 11 laboratories also applied a marker for CK HMW. In this context it should be notified that the p63 clones 4A4 and Y4A3 are not CE IVD labelled but sold for Research-Use-Only purpose. There was no significant difference in the performance and staining results regarding the composition of the PIN-cocktail. The PIN-cocktail reactions were visualized both by a single chromogene (such as DAB) or by a dual chromogene system (DAB and Fast Red). In general it is advisable to use different chromogenes to identify and differentiate the individual reactions in a double staining procedure, but as the markers in a PIN-cocktail are located in different compartments (nuclei versus cytoplasm) in the individual epithelial cells of the prostate, and in different cell layers (luminal versus basal) it is possible to use one chromogene to simplify the protocol set-up.

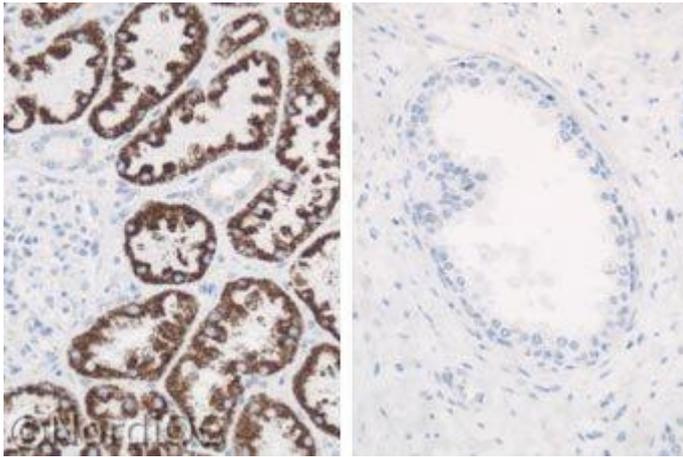
This was the second assessment of AMACR/PIN-cocktail in NordiQC. A constant proportion of sufficient results has been seen as shown in table 2:

Table 2. **Sufficient results with AMACR/PIN-cocktail in the two NordiQC runs**

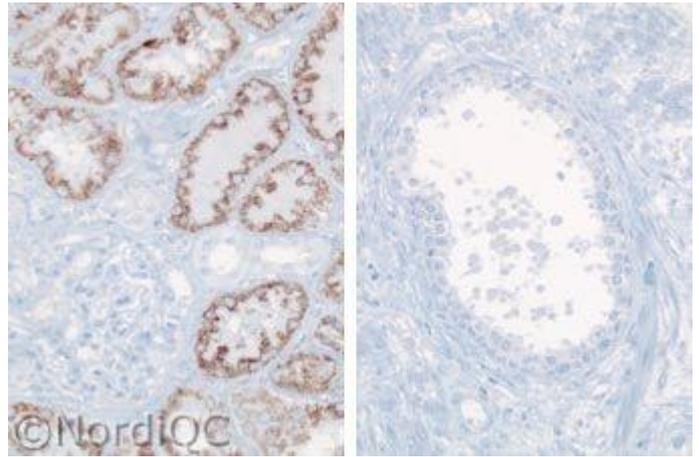
	Run 16 2006	Run 26 2009
Participants, n=	65	106
Sufficient results	89 %	90 %

### **Conclusion**

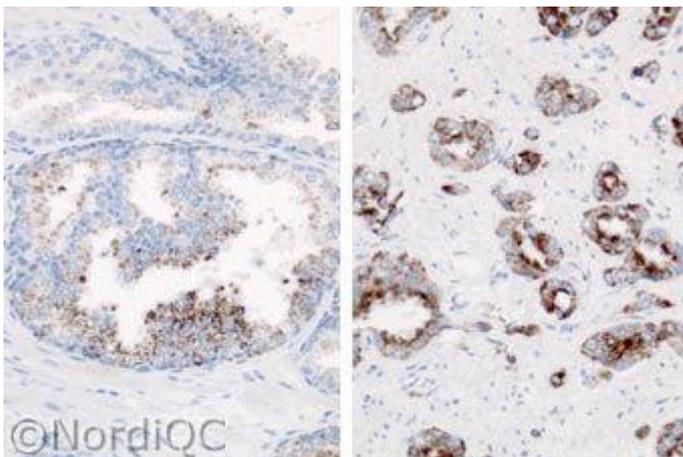
The rmAb clone 13H4 and the pAb p504S, CP200 are both recommendable AMACR Abs. They can be used single or in PIN-cocktails together with, e.g., p63 and CK-HMW. HIER is mandatory for an optimal performance. The AMACR staining must be calibrated carefully. Normal prostate is recommendable as negative control and normal kidney as positive control.



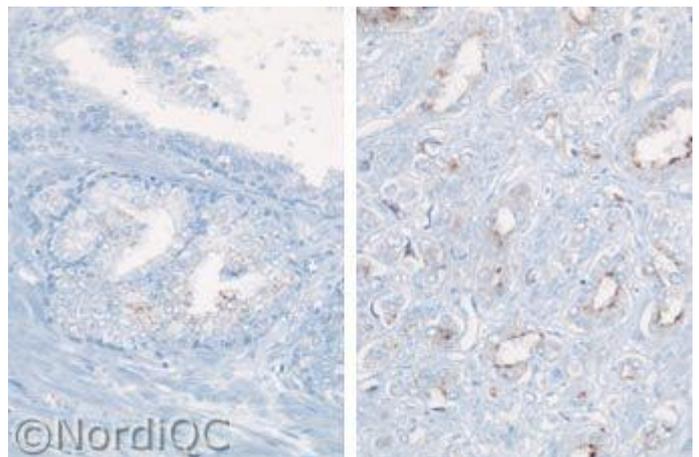
**Fig. 1a**  
 Optimal staining for AMACR using the rmAb 13H4 optimally calibrated and with HIER in an alkaline buffer.  
Left: The epithelial cells lining the proximal tubules show a moderate to strong and granular cytoplasmic staining.  
Right: The epithelial cells of the benign hyperplastic prostatic glands are negative.  
 Also compare with Figs. 2a left & right – same protocol.



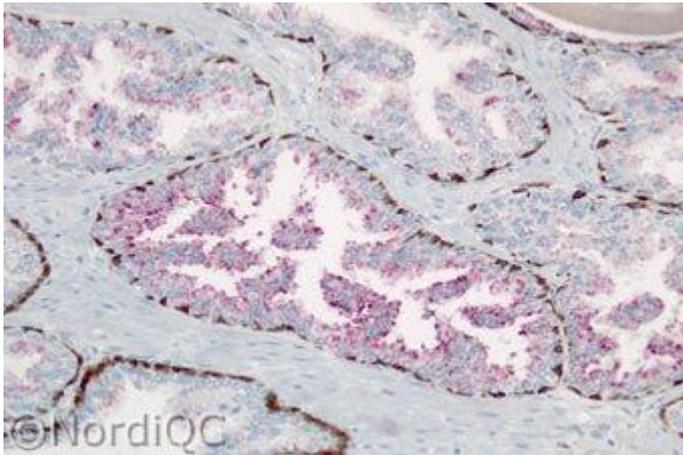
**Fig. 1b**  
 Insufficient staining for AMACR using the rmAb 13H4 too diluted.  
Left: The epithelial cells lining the proximal tubules show only a weak to moderate granular cytoplasmic staining.  
Right: The epithelial cells of the benign hyperplastic prostatic glands are negative.  
 Also compare with Figs. 2b left & right – same protocol.



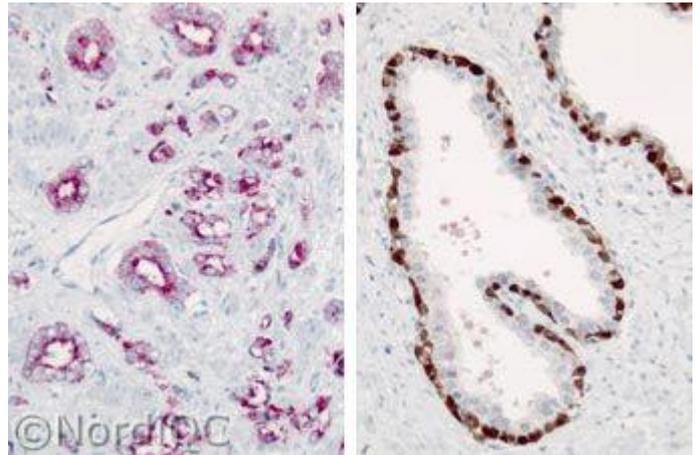
**Fig. 2a**  
 Optimal staining for AMACR using same protocol as in Figs. 1a.  
Left: The majority of the neoplastic cells of the PIN lesion show a distinct granular cytoplasmic staining.  
Right: Virtually all the neoplastic cells of the prostate adenocarcinoma no. 5 show a strong and distinct granular cytoplasmic staining.



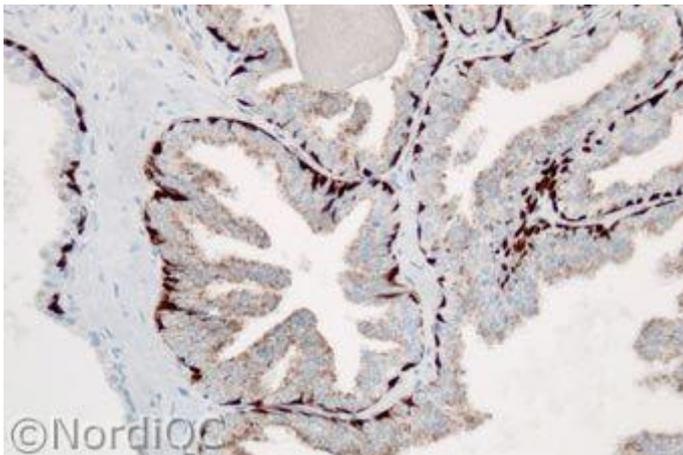
**Fig. 2b**  
 Insufficient staining for AMACR using same protocol as in Figs. 1b. - same fields as in Figs. 2a.  
Left: Only scattered neoplastic cells of the PIN lesion show a weak granular cytoplasmic staining.  
Right: The neoplastic cells of the prostate adenocarcinoma no. 5 are demonstrated, but the intensity and proportion of the positive cells is significantly reduced compared to the result in Fig. 2a right.



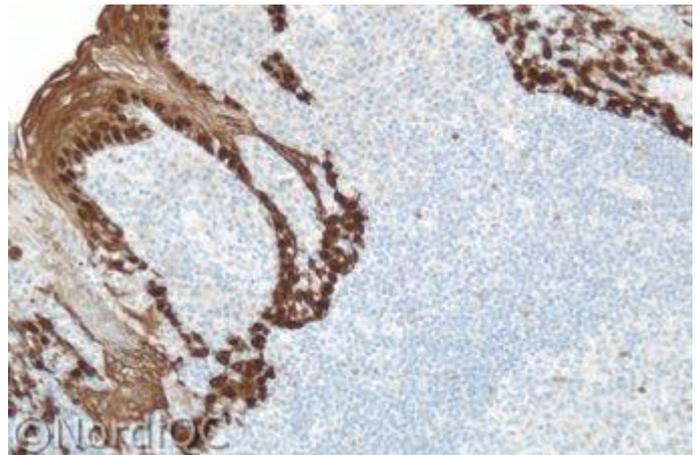
**Fig. 3a**  
Optimal staining of the PIN lesion using a cocktail of the mAb clone 13H4 for AMACR, mAb clone XM26 for CK5 and mAb clone 4A4 for p63 visualized by a dual chromogene based protocol. The neoplastic cells of the PIN lesion show a distinct granular cytoplasmic staining for AMACR, visualized by Fast Red, while the basal cells show a distinct nuclear and cytoplasmic staining for p63 and CK5, visualized by DAB.



**Fig. 3b**  
Optimal staining using same Ab cocktail as in Fig. 3a.  
**Left:** Virtually all the neoplastic cells of the prostate adenocarcinoma no. 5 show a strong and distinct granular cytoplasmic staining.  
**Right:** The basal cells of the hyperplastic prostatic gland show a distinct nuclear and cytoplasmic staining, while the epithelial cells are unstained.



**Fig. 4a**  
Optimal staining of the PIN lesion using a cocktail of the mAb clone 13H4 for AMACR, mAb clone D5 16/B4 for CK5/6 and mAb clone 4A4 for p63, all visualized with the same chromogene and detection system. The neoplastic cells of the PIN lesion show a distinct granular cytoplasmic staining for AMACR, while the basal cells show a distinct nuclear and cytoplasmic staining for p63 and CK5/6, all visualized by DAB.



**Fig. 4b**  
Optimal staining of the tonsil using same Ab cocktail as in Fig. 4a. All the squamous epithelial cells show a moderate to strong cytoplasmic staining for CK5/6, while the basal epithelial cells also show a strong nuclear reaction for p63. In the germinal centre and in the T-zone scattered lymphocytes also show a weak nuclear p63 staining.

SN/HN/MV/LE 3-7-2009